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Monoclonal antibodies against the *Drosophila* nervous system

(neuroimmunology/immunohistochemistry/brain/retina/immunoblots)

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ABSTRACT **A panel of 148 monoclonal antibodies directed against *Drosophila* neural antigens has been prepared by using mice immunized with homogenates of *Drosophila* tissue. Antibodies were screened immunohistochemically on cryostat sections of fly heads. A large diversity of staining patterns was observed. Some antigens were broadly distributed among tissues; others were highly specific to nerve fibers, neuropil, muscle, the tracheal system, cell nuclei, photoreceptors, or other structures. The antigens for many of the antibodies have been identified on immunoblots. Monoclonal antibodies that identify specific molecules within the nervous system should prove useful in the study of the molecular genetics of neural development.**

The development and function of the nervous system involves the participation of various genetically encoded macromolecules whose nature and identities are largely unknown. An incisive approach toward identifying them is offered by the hybridoma technique (1), which can produce monoclonal antibodies (MAbs) against specific molecules singled out from a complex mixture of immunogens. For example, by immunization with leech segmental ganglia, Zipser and McKay (2) found MAbs that recognized antigens expressed in different subsets of neurons. Trisler *et al.* (3) used immunization with chick retinal segments and identified a molecule that occurs in a gradient across the developing retina.

As a step toward elucidating, at the molecular level, the relationship between the genome and the nervous system, we have produced a battery of MAbs showing specificity for the *Drosophila* nervous system and related tissues. *Drosophila* offers the advantage that both classical genetic and recombinant DNA techniques can be used to identify the gene that encodes the antigen. Mutations in the gene that alter or delete the antigen can provide insight into its role in development, physiology, and behavior.

MATERIALS AND METHODS

Generation of MAbs. Hybridomas were obtained from five fusions. Immunogens were homogenates of heads, brains, or retinas dissected from *Drosophila melanogaster* (C-S strain) adult flies previously frozen at −90°C and dehydrated at −20°C in acetone. Each BALB/c mouse received, over a period of 4–20 months, five injections (four intraperitoneal and one intravenous). Each injection contained material dissected from 20–50 flies. Standard procedures using NS-1 myeloma cells (4) were followed to generate hybridomas, which were cloned by limiting dilution. The MAbs described here are IgGs, except 3F12 which is IgM and 3H6 and 4E9 for which the class is undetermined.

Immunohistochemistry. Cryostat sections (2–10 μm) of fly heads were prepared and stored on coverslips at −20°C over silica gel. To eliminate eye pigments, which can cause fluorescent background, *cinnabar brown* mutant flies were used. Staining was done at room temperature. The sections were fixed with 2% formalin in 75 mM Na phosphate buffer (pH 7.0) for 30 min and then rinsed for 5 min in 10 mM Tris·HCl, pH 7.5/130 mM NaCl/5 mM KCl/5 mM NaN_3/1 mM EGTA (TBS). The fixed sections were covered with 50–100 μl of hybridoma supernatant (diluted 1:1 with TBS) in a humidified chamber for 15 min, rinsed for 5 min in TBS, and incubated for 15 min with 50–100 μl of fluorescein isothiocyanate-conjugated goat anti-mouse IgG (H+L) (Cappel) diluted 1:50 with TBS. After a 5-min rinse in TBS, the cover slips were mounted with 90% (vol/vol) glycerol in TBS and viewed by epifluorescence.

Immunoblots. About 60 frozen fly heads were homogenized in a Kontes glass homogenizer in 0.15 ml of $NaDodSO_4$ sample buffer [2.3% $NaDodSO_4$/5% mercaptoethanol/63 mM Tris·HCl, pH 6.8/10% (vol/vol) glycerol], heated (100°C, 3 min), and centrifuged. The entire soluble fraction was loaded into a 6.5-cm-wide slot of a 10% $NaDodSO_4$/polyacrylamide gel and electrophoresed in Tris glycine buffer (5). The gel was electroblotted (6) to nitrocellulose paper by using 50 mM Na borate at pH 8.5, and the immunoblots were processed essentially as described by Matus and co-workers (7, 8). Strips (3 mm wide) were cut, and each was incubated overnight at 22°C with 1 ml of hybridoma supernatant diluted to 3 ml with 1.5× buffer A (50 mM Tris·HCl, pH 7.4/0.2 M NaCl/5% bovine serum albumin/10% calf serum). Strips were washed (three times, 30 min each) in buffer B (50 mM Tris·HCl, pH 7.4/0.2 M NaCl) and incubated with 3 ml of buffer A containing 0.6 μg of ^{125}I-labeled rabbit anti-mouse IgG (H+L) (Cappel) labeled by the IODOGEN technique (9) (3–7.5 × 10^6 cpm/μg of protein), washed as before in buffer B plus 0.5% Nonidet P-40, dried, and autoradiographed.

RESULTS

Diverse Specificities of MAbs. Our experiments were aimed at eliciting immune responses to various components of the adult nervous system, with tissue homogenates used as immunogens. An immunohistochemical screening method with cryostat sections of a pellet of fly heads in embedding medium proved sensitive and provided immediate anatomical localization of each antigen. Of 1,800 hybridoma wells tested, approximately 40% gave positive staining on head sections. Of these,

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Abbreviation: MAb, monoclonal antibody.

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148 were chosen and cloned by limiting dilution. They were chosen to represent diverse patterns of staining—some specific to defined tissues, and others staining more generally (10). When supernatant medium from an NS-1 myeloma culture was used instead of hybridoma supernatant, only the autofluorescence of the fly head and proboscis cuticles were observed (Fig. 1*A*). This was easily distinguished, by its orange color, from the green fluorescence due to fluorescein-conjugated antibody. With hybridoma supernatants, many staining patterns were mutually exclusive, demonstrating that the nonspecific background was low.

Antibodies that stained tissues throughout the entire adult head were commonly encountered. Fig. 1*B* illustrates a generally staining MAb, G11B, which serves to illuminate the various head tissues. A second MAb, 8C5, specifically and intensely stained cell nuclei (Fig. 1*C*). This MAb also stained the nuclei of a wide range of invertebrate, vertebrate, protozoan, and plant cells. Thus, it appears to react with a highly conserved nuclear antigen.

Certain antibodies stained only neural structures in the head, including brain, retina, and nerve (MAb 6B11; Fig. 1*D*). Other MAbs were specific to non-neuronal tissues. MAb 3E2 exclu-

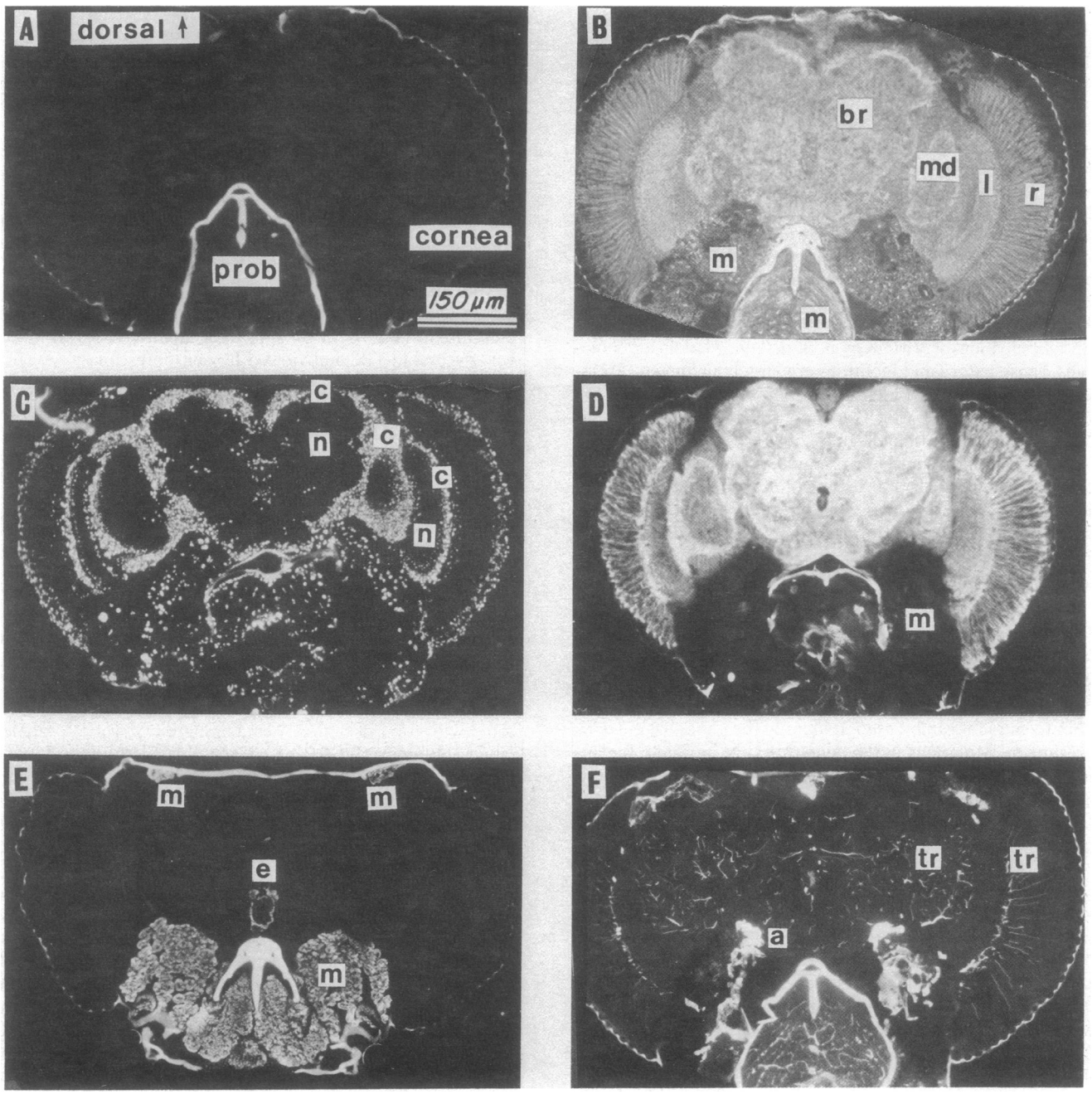

FIG. 1. Immunofluorescent staining of *Drosophila* head sections with MAbs. Plane of section is perpendicular to the body axis. (*A*) Control: supernatant from NS-1 myeloma culture instead of hybridoma supernatant. Note autofluorescence of cornea, proboscis (prob), and dorsal head cuticle; this was easily distinguished by its orange color, in contrast to the green fluorescence of fluorescein. (*B*) General staining of all tissues by MAb G11B. This serves to illustrate the internal anatomy. br, Central brain; l, lamina; m, muscles; md, medulla; r, retina. (*C*) Specific staining of nuclei by MAb 8C5. Note densely packed nuclei in brain cortex (c), in contrast to neuropil (n). (*D*) MAb 6B11 stained retina, brain, and nerves but not muscles. (*E*) MAb 3E2 stained muscles specifically, including proboscis muscles (m), esophagus wall (e), and dorsal muscles (m). (*F*) The tracheal system (tr) is revealed by MAb 10G4. Note two large air sacs (a) dorsolateral to the proboscis.

sively bound to muscles, as evident in Fig. 1*E* which shows staining of the massive proboscis muscles, the two muscles underlying the dorsal cuticle, and the muscular wall of the esophagus which passes through the brain. This MAb also recognized skeletal and visceral muscles in embryos, larvae, and adults, showing cross-striations. Certain MAbs recognized other head structures. For example, MAb 10G4 (Fig. 1*F*) identified the tracheal system, which infiltrates the brain, the retina, and the muscle.

MAb Specificity Within the Nervous System. The antibodies that were positive for the nervous system revealed a complex antigenic organization of the fly brain (Fig. 2). As in other arthropods, the *Drosophila* brain consists of a cellular cortex, nerve tracts, and an inner neuropil of cellular processes and synapses. MAb 2G4B specifically stained the neuropil; the inner nerve tracts were unstained (Fig. 2*A*). Nerve fibers throughout the optic lobes and the brain were specifically marked by MAb 4C6, which produced a pattern resembling that observed with classical reduced silver stains (Fig. 2*B*). This MAb stained nerves in the adult fly, the larva, and the embryo. MAb D12A (Fig. 2*C*) preferentially stained the cortex of the brain and medulla, in a fashion roughly complementary to MAb 2G4B. Note, however, that both antibodies stained the lamina.

A striking example of specificity within the brain is illustrated by MAb 2E6 (Fig. 2*D*). Although this antibody strongly stained the retina and the central region of the brain, the medulla was stained only faintly and the lamina was negative. Thus, the lamina and medulla appear to be antigenically distinct from the rest of the brain.

Immunological Dissection of the Retina with MAbs. The compound eye of *Drosophila* is a hexagonal "neurocrystalline" array (11) of ommatidia, each containing a bundle of eight photoreceptor cells whose axons terminate in a precise pattern of synaptic connections in the lamina and medulla. In addition, each ommatidium contains a lens, a crystalline cone, cone cells which secrete the material in the cone, several classes of pigment cells, and a sensory bristle. The cornea and basement membrane delimit the outer and inner boundaries of the eye. For studying eye development immunological markers for the various retinal components would be useful. Indeed, many of our MAbs stained selectively within the eye. Several examples are illustrated in Fig. 3.

MAbs that label, respectively, the lens, the underlying crystalline cone, and the secretory cone cells are shown in Fig. 3 *A*, *B*, and *C*. MAb 24B10 appeared to be specific for photoreceptor cells and their axons (Fig. 3 *D* and *E*). Of the eight photoreceptor axons from each ommatidium, axons 1–6 are known to terminate in the lamina and axons 7 and 8 terminate in the medulla (12, 13). The precise array of axons seen in the medulla in Fig. 3*D* is consistent with the projections of axons 7 and 8. This MAb also stained the photoreceptors and their axons in the ocelli and the larval photosensitive organ (14). MAb 21A6 (Fig. 3 *F* and *G*) highlighted a structure inside each cluster of photoreceptor cells, extending down to the basement membrane. Note that the underlying lamina and medulla were unstained.

Several of these MAbs have been used to detect the developing photoreceptor cells in the eye imaginal disc, which is attached to the larval brain via the optic stalk. During the third

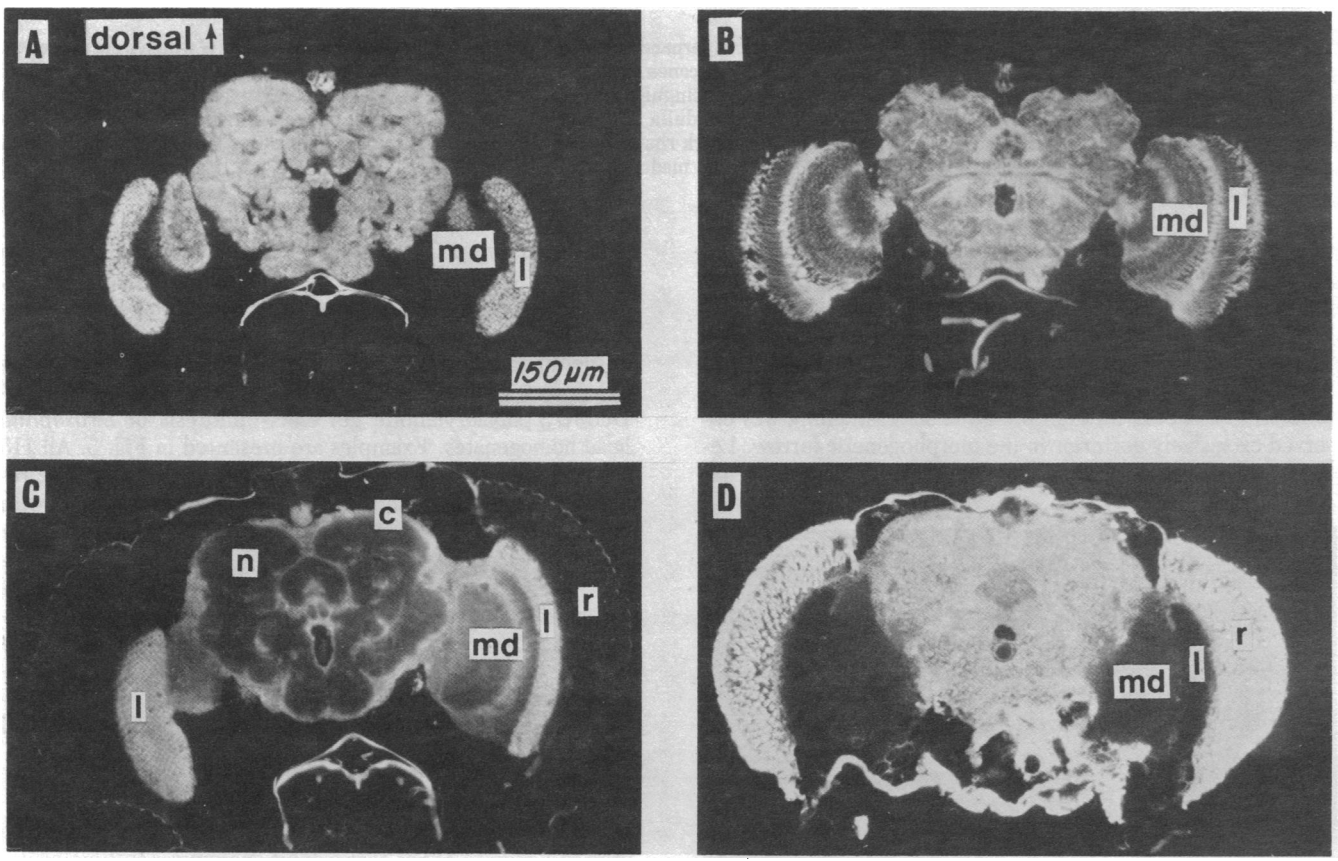

FIG. 2. MAb specificity within the nervous system. (*A*) MAb 2G4B stained neuropil within the central brain, the lamina (l), and the medulla (md). The cortex and retina were negative. (*B*) MAb 4C6 stained nerve fibers throughout the lamina (l), medulla (md), and brain. (*C*) MAb D12A stained the brain cortex (c). Note that neuropil (n) of central brain was dark, in contrast to *A*. In the lamina (l), both neuropil and cortex were strongly stained. The plane of section was slightly oblique, showing highly ordered structure of the lamina on the left. (*D*) Lamina (l) and medulla (md) were unstained by MAb 2E6, although it strongly stained retina (r) and central brain.

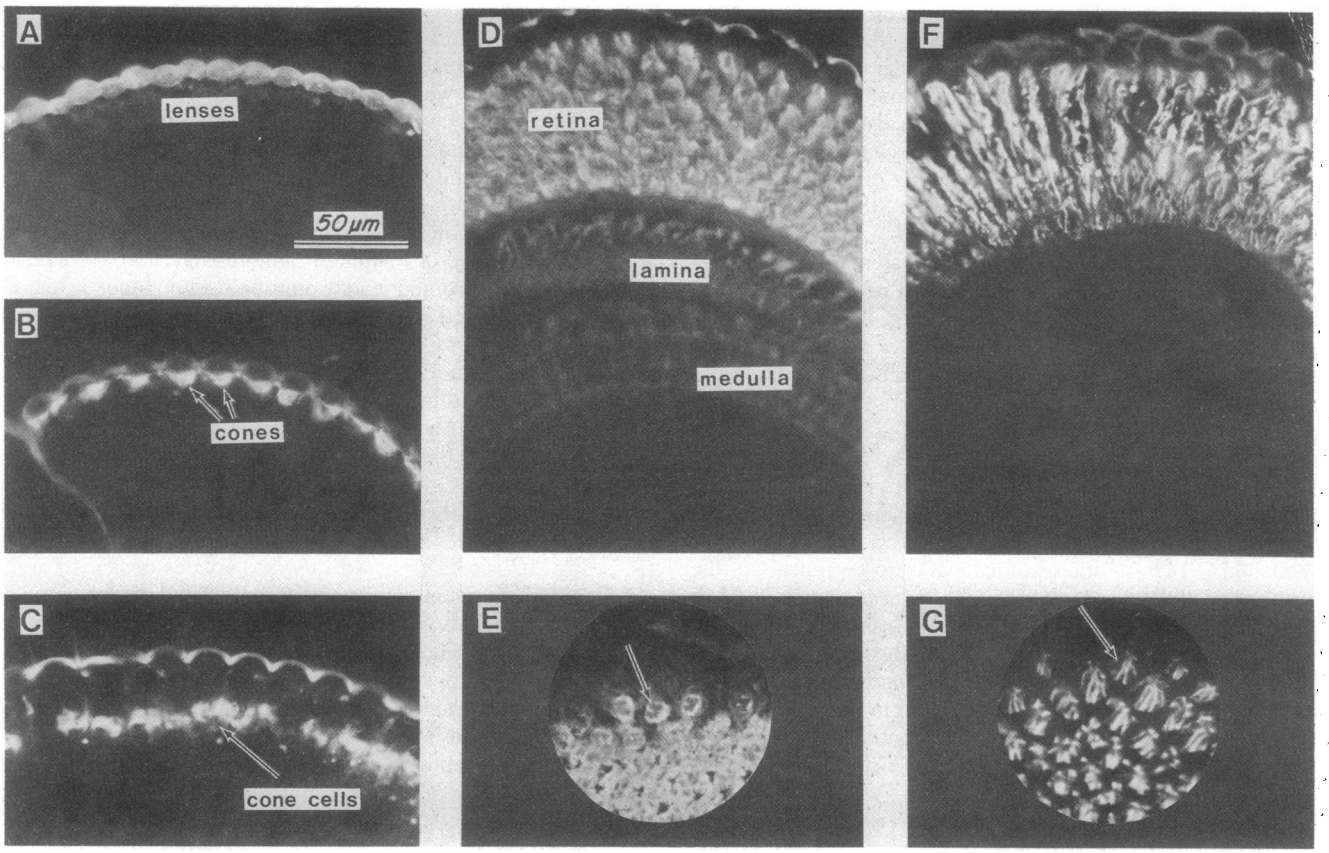

FIG. 3. Dissection of compound eye with MAbs. Sections were cut perpendicular to the surface of the eye, except for *E* and *G* which were tangential to the eye surface. (*A*) Lenses stained by MAb 3F12. (*B*) Crystalline cones stained by MAb 22G8. (Streak at left is cuticle autofluorescence.) (*C*) Cone cells stained by MAb 3F11. Autofluorescence of cornea was distinguished by its orange color (see text). (*D*) MAb 24B10 specifically stained photoreceptor cells and their axons. Note projections in lamina and medulla. (*E*) Section tangential to surface of eye, stained with MAb 24B10. Note brightly fluorescent ring of retinula cells surrounding clusters of dark rhabdomeres (arrow). (*F*) MAb 21A6 stained inside the photoreceptor cell cluster in each ommatidium. Note absence of staining in lamina and medulla. (*G*) Section tangential to surface of eye, stained with MAb 21A6. Note dark rhabdomeres (arrow).

larval instar the disc undergoes a wave of differentiation behind an advancing morphogenetic furrow (11). The newly formed photoreceptor cells send their axons down the optic stalk, into the developing optic lobes. A late third-instar eye disc stained with MAb 24B10 is presented in Fig. 4. Immunofluorescence revealed a regular array of cell clusters in the disc, with nerve processes extending through the optic stalk. Staining was observed exclusively posterior to the morphogenetic furrow. Lebovitz and Ready (15) have also reported MAbs that reveal developing arrays in the eye disc. Wilcox *et al.* (16) identified an antigen restricted to the dorsal compartment of the wing disc. Thus, these MAbs should prove useful in studying gene expression during the development of imaginal discs.

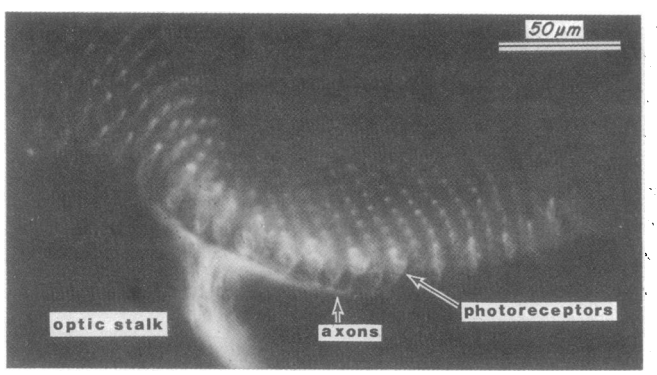

FIG. 4. Developing photoreceptor cell clusters in imaginal eye disc of third-instar larva. Oblique view of whole mount stained with MAb 24B10, seen from posterior aspect. Photoreceptor axons descend into optic stalk on their way to brain.

Identification of Antigens. The molecular specificity of the MAbs was studied with the immunoblot technique and Na-DodSO_4/polyacrylamide gel electrophoresis of *Drosophila* head homogenates. Examples are presented in Fig. 5. All 115 MAbs from fusions 1–3 were tested, and 51 gave positive results. Of these, 25 bound to single bands and 26 reacted with multiple bands or smears. Hawkes *et al.* (8) reported similar results for MAbs to synaptosomal antigens. Many of the antigens were inactivated by proteinase K, suggesting a proteinacious nature (data not shown). There was no obvious correlation between the tissue distribution of an antigen and its immunoblot pattern. Among MAbs that recognized single bands, some exhibited general tissue staining and others exhibited highly specific tissue staining; the same was true for the multiple band group.

DISCUSSION

The results show that whole homogenates of *Drosophila* can be used to generate MAbs that exhibit anatomical and molecular specificity. The wide range of staining patterns obtained suggests that the immune response of the mouse against *Drosophila* tissues is not directed solely against a small number of immunodominant antigens. Within the nervous system, certain antigens were shown to be widely distributed whereas others were

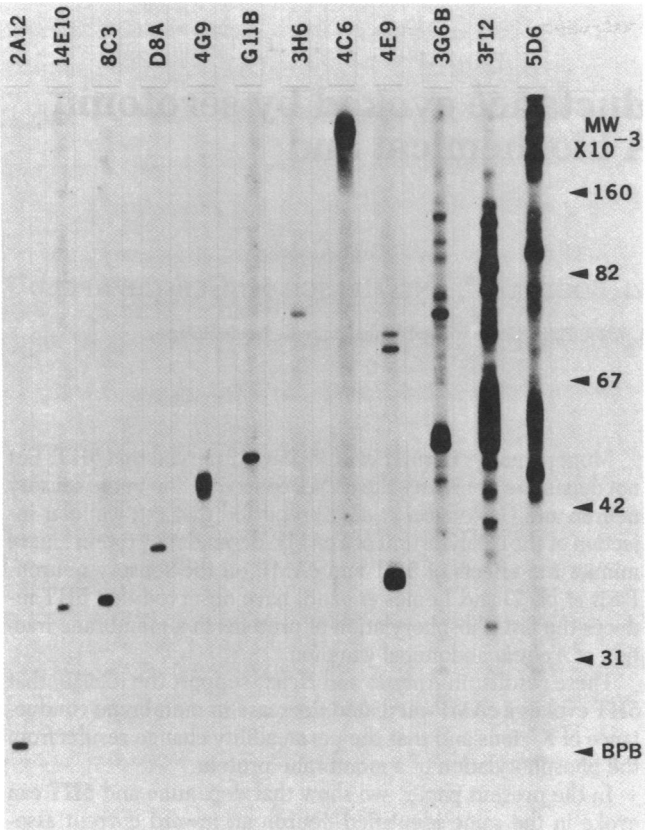

FIG. 5. Immunoblot analysis with *Drosophila* MAbs. A homogenate of *Drosophila* heads was electrophoresed on a polyacrylamide gel and electroblotted to nitrocellulose; the nitrocellulose was cut into strips, and each strip was stained with a different MAb. Autoradiograms showed binding of ^{125}I-labeled second antibody. Predominant staining patterns of these MAbs within the head were: general, G11B, 3G6B; nuclei, 14E10; muscle, 4E9; nervous system, 2A12, 5D6; brain, D8A; neuropil, 8C3, 4G9, 3H6; nerve fibers, 4C6; compound eye lenses, 3F12. Molecular weight markers: carbonic anhydrase (30,000), actin (42,000), bovine serum albumin (67,000), *E. coli* RNA polymerase σ-subunit (82,000), and *E. coli* RNA polymerase β-subunit (160,000). BPB: bromphenol blue tracking dye.

specific to neuropil, cortex, or nerve fibers. Antigenic differences between parts of the brain were also revealed—e.g., Fig. 2*D* shows the optic lobes to be deficient in an antigen that is abundant in the central region of the brain. A subset of the MAbs served to distinguish various components of the eye. MAbs isolated in other laboratories also produce varied staining patterns in the *Drosophila* nervous system (15, 17).

In the immunoblot analysis, approximately one-fifth of the 115 MAbs tested revealed single antigen bands. A similar number bound to multiple bands, and the rest gave none. Multiple bands could be due to a single antigenic determinant being shared by different molecules (e.g., the carbohydrate moieties of glycoproteins), to proteolysis of the antigen, or to a single antibody recognizing more than one antigenic determinant. Possible reasons for the null immunoblots may be irreversible denaturation of the antigenic site by $NaDodSO_4$, low affinity of the antibodies, inability of the antigen to enter the gel, or failure of the antigen to bind to nitrocellulose. These antigens may be identifiable by other techniques.

Development of the nervous system involves cell differentiation and the formation of an intricate pattern of connectivity, which ultimately gives rise to behavior. MAbs can be used to reveal which gene products are present at a given time. Because many of the antigens are polypeptides, recombinant DNA technology may be applied to MAbs to obtain the corresponding cDNAs which then can be used to locate the genes on the *Drosophila* genome. Mutations in these genes can be generated to identify their functions. The isolation of a panel of *Drosophila* MAbs should be useful in approaching the genetic and molecular mechanisms involved in neural differentiation.

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